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Review

An update on vitamin D signaling and cancer

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ABSTRACT

A low vitamin D status is associated with an increased risk of various cancers, such as of colon, breast, prostate and hematological cells. The biologically most active vitamin D metabolite 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is a high affinity ligand of the transcription factor vitamin D receptor (VDR). $1,25(OH)_2D_3$ induces *via* VDR changes to the epigenome of healthy and neoplastic cells and in this way influences their transcriptome. Ligand-activated VDR binds to more than 10,000 loci within the human genome and affects the transcription of some 1000 target genes in a large proportion of human tissues and cell types. From the evolutionary perspective, the prime role of vitamin D was probably the control of energy metabolism later shifting to modulate innate and adaptive immunity as well as to regulate calcium and bone homeostasis. Since rapidly growing immune and cancer cells both use the same pathways and genes for controlling their proliferation, differentiation and apoptosis, not surprisingly, vitamin D signaling changes these processes also in neoplastic cells. Thus, anti-cancer effects of vitamin D may derive from managing growth and differentiation in immunity. This review provides an update on the molecular basis of vitamin D signaling, *i.e.*, the effects of $1,25(OH)_2D_3$ on the epigenome and transcriptome, and its relationship to cancer prevention and therapy.

1. Introduction

Cancer is the overarching term describing a multitude of very heterogenous diseases that have in common displaying uncontrolled overgrowth of cells in any tissue of an individual [1]. The molecular basis of cancer is the accumulation of point mutations and copy number

Abbreviations: $1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3, also called calcitriol; $25(OH)D_3$, 25-hydroxyvitamin D_3, also called calcidiol; AML, acute myeloid leukemia; AMPK, adenosine monophosphate-activated protein kinase (official gene symbol: *PRKAA1*); APC, APC regulator of WNT signaling pathway; AXIN2, axin 2; BCL2, BCL2 apoptosis regulator; BRCA1, BRCA1 DNA repair associated; BRD7, bromodomain containing 7; CAF, cancer-associated fibroblast; CCND1, cyclin D1; CDH, cadherin; CDK, cyclin dependent kinase; CDKN, cyclin dependent kinase inhibitor; CEBPA, CCAAT/enhancer binding protein alpha; ChIP-seq, chromatin immunoprecipitation sequencing; CRC, colorectal cancer; CST5, cystatin D; CTCF, CCCTC-binding factor; CTNNB1, catenin beta 1; CXCL8, C-X-C motif chemokine ligand 8, also called IL8; CYP24A1, cytochrome P450 family 24 subfamily A member 1; DKK1, Dickkopf WNT signaling pathway inhibitor 1; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, estrogen receptor (official gene symbols: *ESR1* and *ESR2*); FOS, Fos proto-oncogene, AP-1 transcription factor subunit; GABPA, GA binding protein transcription factor subunit alpha; HER2, human epidermal growth factor receptor 2 (official gene symbol: *ERBB2*); HOXA9, homeobox A9; ID1, inhibitor of DNA binding 1, HLH protein; IFNG, interferon gamma; IGF, insulin-like growth factor; IL, interleukin; JAK, Janus kinase; JUN, Jun proto-oncogene, AP-1 transcription factor subunit; KDM6B, lysine demethylase 6B; LGR5, leucine rich repeat containing G protein-coupled receptor 5; MAPK14, mitogen-activated protein kinase 14; MECOM, MDS1 and EVI1 complex locus; MEIS1, meis homeobox 1; miR, microRNA; MMP9, matrix metalloprotease 9; MYC, MYC proto-oncogene, BHLH transcription factor; ncRNA, non-coding RNA; NF, normal fibroblast; PBMC, peripheral blood mononuclear cell; Pol II, RNA polymerase II; PR, progesterone receptor (official gene symbol: *PGR*); PU.1, purine-rich box 1, Spi-1 proto-oncogene (official gene symbol: *SPI1*); PTGER2, prostaglandin E receptor 2; PTGS2, prostaglandin-endoperoxide synthase 2, also called COX2; PTH, parathyroid hormone; RHOA, Ras homolog family member A; RNA-seq, RNA sequencing; RNF43, ring finger protein 43; ROCK1, Rho associated coiled-coil containing protein kinase 1; RPS6KA5, ribosomal protein S6 kinase A5, also called MSK1; RSPO2, R-spondin 2; RXR, retinoid X receptor; SERPINE1, serpin family E member 1, also called plasminogen activator inhibitor (PAI); SNAI, snail family transcriptional repressor; SPRY2, sprouty RTK signaling antagonist 2; STAT, signal transducers and activators of transcription; TAD, topologically associated domain; TCF7L2, transcription factor 7 like 2; TGFBR1, transforming growth factor beta receptor 1; T_H, T helper; TIMP1, TIMP metallopeptidase inhibitor 1; TLR, Toll-like receptor; TNBC, triple-negative breast cancer; TNF, tumor necrosis factor; TNC, tenascin C; T_{reg}, regulatory T; TSS, transcription start site; VDR, vitamin D receptor; VEGFA, vascular endothelial growth factor A; WNT3A, WNT family member 3A

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variations, such as amplification and deletions or big chromosomal alterations like translocations and aneuploidies, that enhance the activity of oncogenes and decrease that of tumor suppressor genes [2,3]. These genomic instabilities are modulated by epigenetic changes through direct actions of chromatin modifying enzymes as well as *via* indirect effects of transcription factors [4]. Both chromatin modifiers and transcription factors are often found at the endpoint of signal transduction cascades that are stimulated by various intra- and extracellular signals. Changes of the epigenome are triggered by signals of the cellular environment, such as nutrients, toxins and inflammation-related cytokines and chemokines [5]. Thus, epigenetic changes can have both detrimental as well as beneficial effects on cancer onset and progression.

The worldwide increase in life expectancy raises the rates of cancer morbidity and mortality, with some 18 million new cancer cases and nearly 10 million cancer deaths in 2018 [6]. The costly treatment of the disease, such as surgery, radiation therapy and chemotherapy, is a financial burden for the healthcare systems of developed countries and is even not easily accessible to the majority of the population in developing countries [7]. Thus, there is urgent need for anti-cancer options that combine preventive with therapeutic potentials, ideally at low cost.

In this context vitamin D_3 (also called cholecalciferol) has gained significant attention as an inexpensive and readily accessible natural compound [8]. The main source of this seco-steroid is its non-enzymatic production in human skin from UV-B exposed 7-dehydrocholesterol, which is an abundant direct cholesterol precursor (Fig. 1A) [9]. Circulating vitamin D_3 from endogenous production as well as from dietary uptake is converted in the liver to 25-hydroxyvitamin D_3 ($25(OH)D_3$, also called calcidiol). The serum concentration of this most stable vitamin D metabolite is used as biomarker for the vitamin D status of a person [10]. The natural way of producing vitamin D_3 *via* UV-B exposure of the skin should be used with precaution, since excessive sun bathing leads to sunburn and may cause different types of skin cancer. Unfortunately, only a limited number of foods, such as the fatty fishes salmon, tuna and mackerel, contain substantial amounts of vitamin D_3. Moreover, only in a few countries foods, such as milk, dairy products, orange juice and margarines, are regularly fortified with vitamin D_2 (also called ergocalciferol) or vitamin D_3 [11]. This makes direct supplementation with vitamin D_3 (800–4000 IU, *i.e.*, 20 − 100 μg/day) an alternative strategy for optimizing the vitamin D status to $25(OH)D_3$ serum levels of 75–150 nM (*i.e.*, 30 − 60 ng/mL) [12].

The hydroxylation of $25(OH)D_3$ at carbon 1, taking place primarily in the kidneys and also in several types of epithelial and immune cells, leads to $1,25(OH)_2D_3$ (also called calcitriol, Fig. 1A), which is the high-affinity ligand of the transcription factor VDR [13]. In this way, in the nucleus vitamin D has a direct effect on gene regulation and *via* the actions of VDR it also affects the epigenome (Fig. 1B) [14]. Moreover, in a variable cell-type and -context a proportion of VDR molecules locate in the cytosol, where they mediate ligand-dependent, rapid modulatory effects on signaling pathways affecting enzymes, kinases, phosphatases and ion channels. These effects do not require changes in gene transcription and are called non-genomic actions [15]. Thus, vitamin D_3 and its metabolites may be ideal compounds for modulating intracellular pathways with impact on cellular growth, differentiation and apoptosis.

This review will provide an update of excellent previous surveys [16–18] on the molecular and cellular basis of vitamin D signaling and its relationship to cancer prevention and therapy.

2. Linking vitamin D with cancer prevention

Already 40 years ago, an epidemiological study suggested that vitamin D may be protective against colorectal cancer (CRC), since increased sun (UV-B) exposure as well as a life at lower latitudes (both causing higher vitamin D_3 formation) leads to a lower incidence for this type of cancer [19]. Interestingly, at about the same time $1,25(OH)_2D_3$ was found to have *in vitro* an anti-proliferative effect on melanoma cells [20]. In general, a low vitamin D status seems to be associated with a higher cancer incidence [21]. A number of vitamin D intervention trials with different types of cancer, but not all, confirmed this observation and most convincing data were presented for CRC [22–24]. Moreover, there are many *in vitro* studies and a few *in vivo* data indicating that vitamin D is also effective against breast and prostate cancer as well as leukemia and lymphoma. A final answer concerning the impact of vitamin D on cancer prevention was expected from randomized control trials.

The large randomized control trial VITAL [25] had enrolled 25,871 participants without a history of cancer and used 2000 IU (50 μg) vitamin D_3 per day for primary prevention of cancer within a period of 5 years. Although the primary analysis of that study did not report any significant reduction in the risk of cancer mortality, a secondary analysis suggested a benefit of vitamin D_3 after exclusion of early follow-up data and for subgroups of individuals in cancer incidence and/or mortality [25,26]. Similarly, a meta-analysis [27] of three other randomized control trials [28–30], each of them having a null result, found that vitamin D_3 supplementation leads to a significantly lower total cancer mortality. In this context, the concept of a personalized vitamin D response index [31] may be taken into account. As shown with Finnish cohorts [32,33] about 25 % of the population seem to be low responders to vitamin D supplementation, *i.e.*, these persons need higher daily doses of vitamin D_3, in order to reach the full clinical benefit. So far, participants of randomized control trials have not been stratified for low, mid and high vitamin D responders. Moreover, many study subjects were recruited with a rather high vitamin D status, *i.e.*, they may have been in the range of vitamin D sufficiency and did not need further supplementation.

In summary, a stratification of the participants, a better design including the analysis of health outcomes in relation to changes in individual serum $25(OH)D_3$ values and an adequate duration of the study/follow-up as well as the type of statistical analysis majorly influence the final conclusion taken from randomized control trials on vitamin D [34].

3. Vitamin D and its receptor

The 48 nuclear receptors encoded by the human genome form an unusual superfamily of transcription factors, since the majority of these proteins can be activated by small lipophilic molecules in the range of the molecular mass and volume of cholesterol [35]. Prominent members of the superfamily are the endocrine receptors for estrogen, progesterone and cortisol (estrogen receptors (ERs), progesterone receptor (PR) and glucocorticoid receptor) as well as the adopted orphan receptors for fatty acids and oxysterols (peroxisome proliferator-activated receptors and liver X receptors) [36]. Nuclear receptors are characterized by a structurally conserved ligand-binding domain [37]. In case of VDR, some 40 mostly non-polar amino acids within this domain form a ligand-binding pocket that fixes $1,25(OH)_2D_3$ or its synthetic analogs with high specificity [38]. Accordingly, VDR is the only protein that binds at sub-nanomolar concentrations (K_D 0.1 nM) the biologically most active form of vitamin D_3 [39], *i.e.*, VDR is the exclusive mediator of the effects of physiological concentrations of $1,25(OH)_2D_3$.

The high-affinity $1,25(OH)_2D_3$-binding VDR protein evolved already some 550 million years ago in a boneless vertebrate [40]. The initial function of VDR was most likely the regulation of energy metabolism [41]. The fact that the innate immune system and the developing adaptive immunity require substantial amounts of energy [42] seem to have served as a starting point how vitamin D and its receptor got *via* the control of immunometabolism a modulatory impact on immunity [43]. Since immune cells are the most rapidly growing cells of the body, the functions of vitamin D also extended to the control of cellular proliferation, differentiation and apoptosis [44]. Some 400 million years ago some fish species left the calcium-rich ocean and

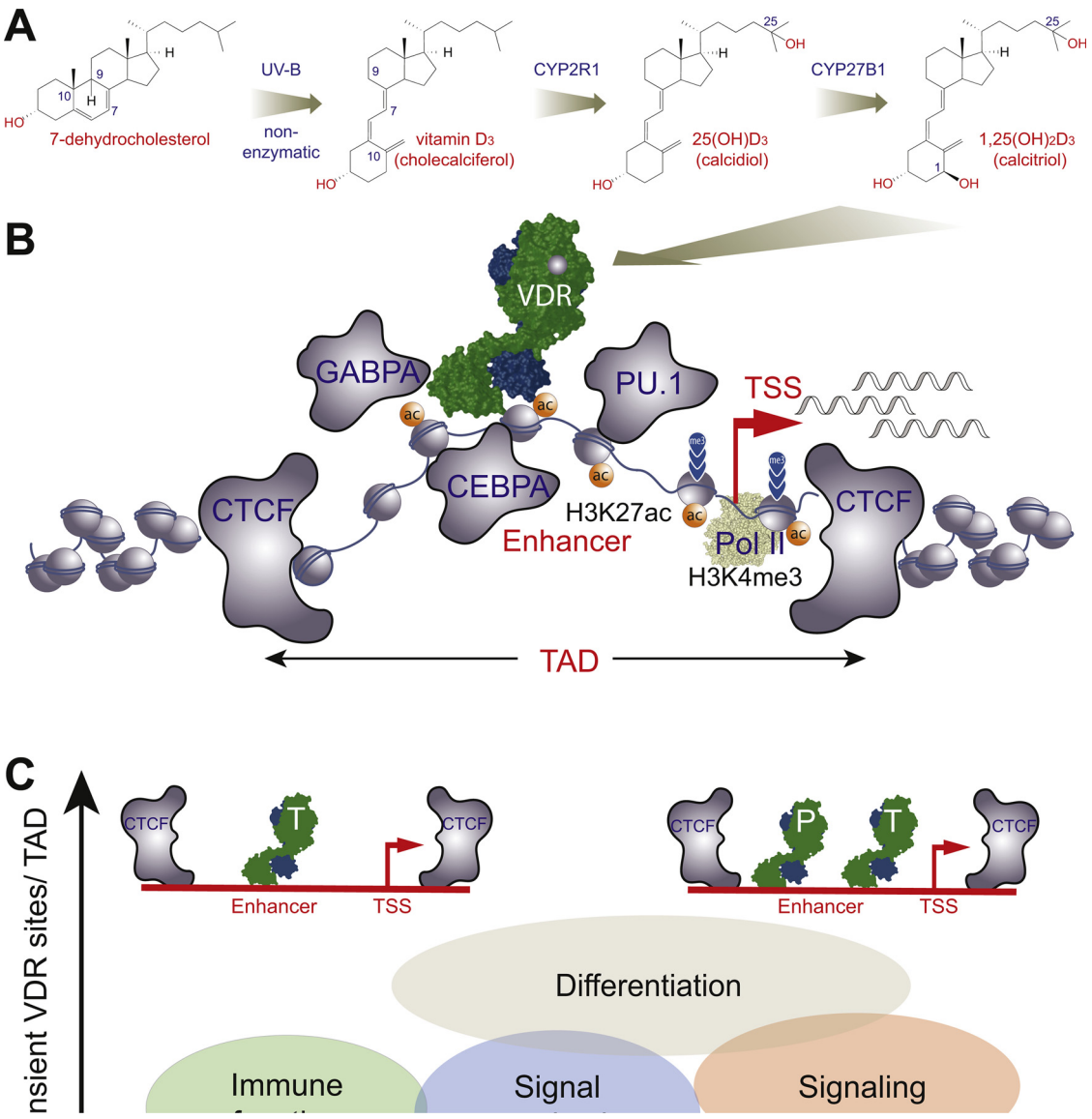

Fig. 1. Vitamin D signaling. Production of vitamin D_3 and its metabolites 25(OH)D_3 and 1,25$(OH)_2D_3$ (A). VDR (green) binds accessible genomic DNA in complex with a partner protein (RXR or others, blue) (B). VDR's DNA binding is supported by the pioneer factors PU.1, CEBPA and/or GABPA. The genomic region that can be influenced by 1,25$(OH)_2D_3$ (*via* binding to VDR) is restricted by CTCF proteins defining left and right TAD borders. Schematic representation of a Voronoi tessellation [55] displaying five TAD classes of the most prominently enriched biological processes (C). The most relevant attributes are the number of persistent and transient VDR sites and were chosen for the x and y axis, respectively.

populated the calcium-poor land, where they were exposed to gravitation making a more stable skeleton essential [45]. At that time vitamin D and VDR gained the additional role of regulating calcium homeostasis, which is indispensable for proper bone formation. In this physiological function vitamin D obtained key importance [46], *i.e.*, in contrast to its previous tasks no other regulatory molecules are able to replace vitamin D. This explains why the prime phenotype of vitamin D deficiency is bone malformation, such as occurring in rickets [47]. Nevertheless, the rather ubiquitous expression of the *VDR* gene in more than half of the 400 human tissues and cell types (www.proteinatlas.org/ENSG00000111424-VDR/tissue) indicates that vitamin D and its receptor have a wider physiological role than the regulation of calcium homeostasis [48].

The VDR cistrome, *i.e.*, the genome-wide binding pattern of the transcription factor, is determined *via* the next-generation sequencing method chromatin immunoprecipitation sequencing (ChIP-seq) [49]. This cistrome had been obtained in immortalized primary human cell types, such as B lymphocytes (GM10855, GM10861 and cells from 30 other HapMap individuals) [50,51], hepatic stellate cells (LX2) [52] and prostate epithelial cells (RWPE1) [53], as well as in cell lines derived from acute monocytic leukemia (AML) of a 1-year-old male (THP-1) [54,55] and CRC of a 58-year-old female (LS180) [56]. In addition, VDR ChIP-seq had been performed with epithelial stem cell-derived organoids of normal tissue of CRC patients [57] as well as with primary normal fibroblasts (NFs) of healthy donors [58] and kidney cortex of human cadavers [59]. From all investigated cellular models, leukemia cells (THP-1) have been studied most extensively for the molecular mechanisms of vitamin D signaling [60]. VDR binding sites drastically up-regulating the gene *CYP24A1* (cytochrome P450 family 24 subfamily A member 1) in THP-1 cells, normal organoids of three different pairs of CRC patients and LS180 cells are displayed as a representative example (Fig. 2A). CYP24A1 is the key vitamin D catabolizing enzyme and regulates the levels of 25(OH)D_3 and 1,25$(OH)_2D_3$ [61], the expression of which is mostly negatively associated with cancer prognosis [62].

Depending on the cell type, the VDR cistrome comprises many

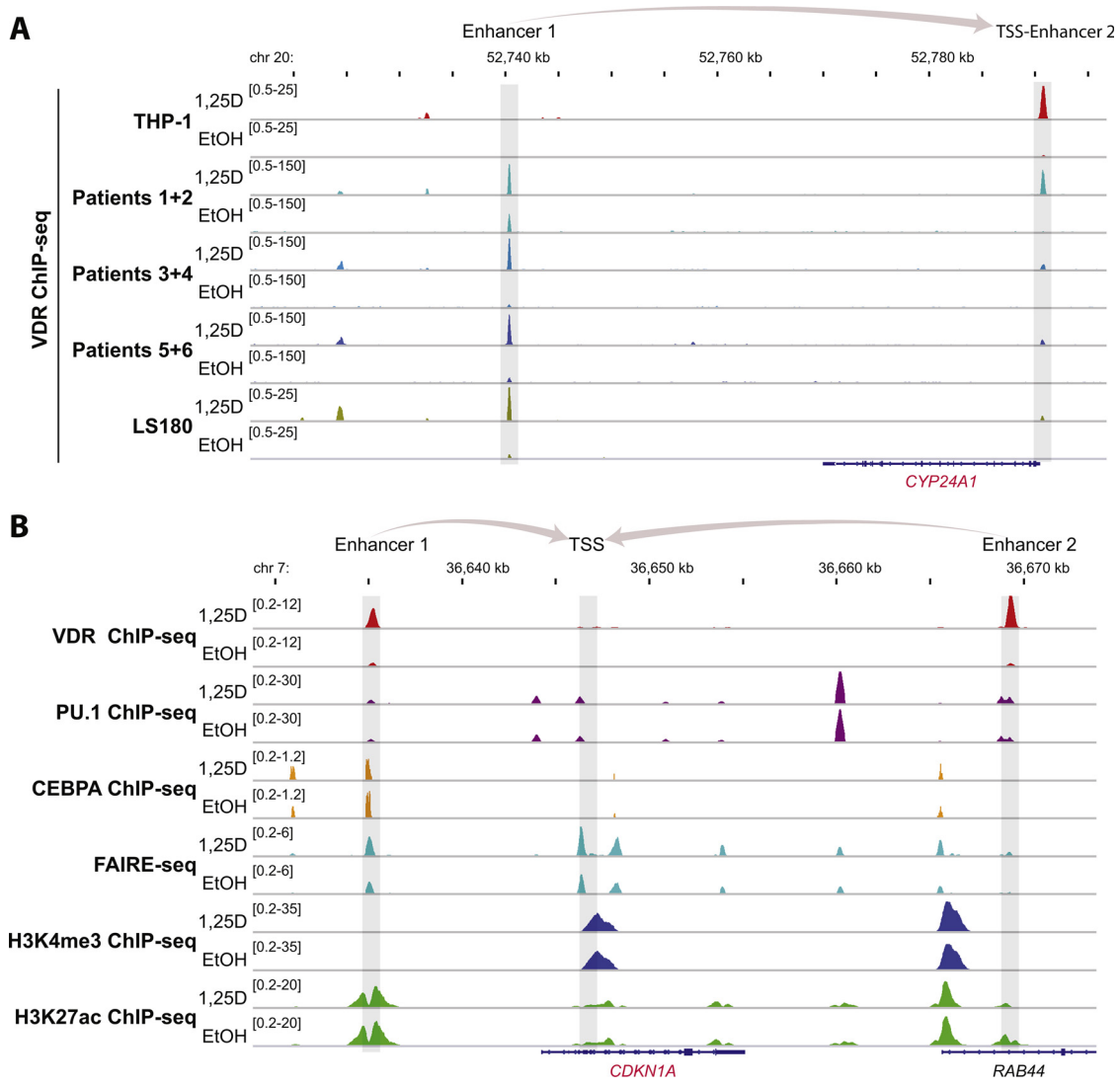

Fig. 2. VDR binding and epigenomic profiles in the region of vitamin D target genes. The IGV browser [201] was used to display the epigenomic profiles at enhancer and TSS regions of the vitamin D target genes *CYP24A1* (A) and *CDKN1A* (B). VDR ChIP-seq data are derived from single experiments performed with organoid cultures of three pairs of CRC patients [57] or cultured LS180 cells [56]. For THP-1 cells the peak tracks display merged data from three biological repeats of ChIP-seq experiments with antibodies against VDR [55], PU.1 [84], CEBPA [85], H3K4me3 [80] and H3K27ac [80] as well as FAIRE-seq data [79] treated for 24 h with $1,25(OH)_2D_3$ (1,25D) or vehicle (EtOH). The gene structures are shown in blue and vitamin D target genes are indicated in red.

thousand VDR binding sites spreading tissue-specifically over the human genome [63]. This is the most likely explanation why cell types differ majorly in their set of vitamin D target genes [64–66]. However, one common observation in all cell types is that a stimulation with $1,25(OH)_2D_3$ leads to a 2- to 10-fold increase in genome-wide VDR binding events [63]. In an average normal or malignant human cell, the VDR cistrome is formed by some 10,000 genomic loci. This is far more than the average number of vitamin D target genes, which is in the order of 200–1000 genes per cell type [57,64–67], *i.e.*, not all VDR binding sites seem to have an impact on gene regulation. However, there are a few hundred VDR loci, on which after ligand stimulation receptor occupancy changes significantly over time while they are always bound to their loci [55]. These persistent VDR binding sites serve as primary contact points for the communication of vitamin D with the human genome, *i.e.*, they act as "hotspots" of vitamin D signaling. In contrast, other VDR sites are only transiently occupied or used exclusively in later steps of the gene activation cascade.

Taken together, vitamin D-triggered binding of VDR to the human genome is the most pronounced and well-understood epigenome-wide effect of $1,25(OH)_2D_3$ [14].

4. Epigenetic basis of vitamin D signaling

Chromatin is a complex of genomic DNA and nucleosomes, which are formed by histone proteins [68]. The epigenome of a cell represents the genome-wide information included in covalent and structural modifications of its chromatin [69]. The epigenome controls the access of transcription factors to their genomic binding sites [70] *via* changes in i) cytosine methylation, in particular at the sites of CpG islands, ii) more than 100 different types of post-translational modifications of histone proteins and iii) the 3-dimensional (3D) organization of the nucleus, such as topologically associated domains (TADs) [71]. For example, well-known markers for active chromatin are acetylated histone H3 proteins at position lysine 27 (H3K27ac), while active transcription start site (TSS) regions are marked by H3K4me3 modifications [72].

The main regulatory regions of a gene are its TSS as well as the binding sites for transcription factors, referred as enhancers, which are found in a Gaussian-type distribution both up- and down-stream of the TSS (Fig. 1B) [73]. The expression of a vitamin D target gene is critically dependent on whether the TSS and at least one VDR-binding

enhancer are located within accessible chromatin [74]. However, more than 90 % of the genomic DNA of a differentiated cell is not accessible, *i.e.*, chromatin largely acts as an intrinsic repressor of gene expression [75,76]. Thus, the so-called "epigenetic landscape" of a differentiated cell type is restricted to some 100–200,000 genomic loci, most of which represent TSS and enhancer regions [73]. Interestingly, the transformation of a normal cell into a cancer cell includes multiple changes in this epigenetic landscape [77]. This de-differentiation process results in an increased number of accessible chromatin regions [78]. Importantly, the epigenome can be modulated by environmental signals, such as $1,25(OH)_2D_3$, *i.e.*, in contrast to the genome the epigenome is dynamic. This implies the potential that vitamin D can re-arrange the epigenome of a tumor cell or its microenvironment [17].

The stimulation of leukemia cells (THP-1) with $1,25(OH)_2D_3$ induces significant changes in the accessibility of their epigenome affecting nearly 9000 of 62,000 open chromatin loci [79]. Similarly, in the same cell line 550 of 22,998 genomic regions with H3K4me3 modifications and 2473 of 45,578 regions with H3K27ac marks respond significantly to a stimulation with $1,25(OH)_2D_3$ [80]. Thus, vitamin D is able to modulate the activity of the epigenome of a cancer cell at more than 500 promoter regions and some 2500 enhancer regions. VDR binding was detected at some 25 % of these chromatin regions, while the remaining $1,25(OH)_2D_3$-triggered sites of the epigenome seem to represent secondary effects of the nuclear hormone. VDR interferes with chromatin modifying proteins, such as KDM6B (lysine demethylase 6B), and the chromatin remodeler BRD7 (bromodomain containing 7). This happens *via* direct and indirect interactions of VDR with these proteins, such as being part of the same large protein complex in the nucleus [81,82] or the regulation of the genes encoding them [83], respectively.

Like most other regular ("settler") transcription factors, VDR functionally interferes with "pioneer" transcription factors, such as PU.1 (purine-rich box 1) [84], CEBPA (CCAAT/enhancer binding protein alpha) [85] and GABPA (GA binding protein transcription factor subunit alpha) [86] (Fig. 1B). Pioneer factors help regular transcription factors to access their genomic binding sites, *i.e.*, in general they amplify the action of settler transcription factors [87]. For example, PU.1 and VDR work together in the process of monocyte and granulocyte differentiation during hematopoiesis [88]. In THP-1 cells on 2/3 of all VDR binding sites also PU.1 is found [84]. Thus, on the genome-wide level PU.1 shows a far higher rate of co-location with VDR as its traditional DNA-binding partner retinoid X receptor (RXR) [63].

Interestingly, at a subset of some 5% of all PU.1 sites $1,25(OH)_2D_3$ significantly supports the DNA binding of the pioneer factor, *i.e.*, VDR can also act as an pioneer for PU.1 [84]. A similar functional interference of vitamin D signaling was observed with the chromatin organizing protein CTCF (CCCTC-binding factor). In THP-1 cells, 1321 of the in total 23,658 CTCF binding sites are significantly affected by a stimulation with $1,25(OH)_2D_3$ [79,89]. CTCF is one of the key proteins organizing the 3D structure of chromatin *via* binding to TAD anchors [90]. The anchors of these chromatin loops act as insulators towards neighboring TADs and are often conserved between tissues and species [91,92]. Vitamin D-sensitive CTCF sites affect the activity of some 600 TADs and the vitamin D target genes included in them (Fig. 1B). Since the human genome is segregated into approximately 3000 TADs [93], some 20 % of all TADs seem to be sensitive to vitamin D.

In summary, vitamin D influences the epigenome not only *via* changes of the chromatin accessibility status affecting VDR and pioneer factor binding, but also on the level of 3D chromatin structure.

5. The vitamin D transcriptome and cancer

Primary vitamin D target genes are modulated in their expression, *i.e.*, in most cases they are up-regulated, when they co-locate on the same TAD with an enhancer region carrying a persistent and/or transient VDR binding site (Fig. 1C) [55]. We define primary vitamin D target as those genes that are significantly up- or down-regulated within 4 h after stimulation with $1,25(OH)_2D_3$ [79]. VDR-activated enhancers activate *via* DNA looping RNA polymerase II (Pol II) on TSS regions leading to a stimulation mRNA production. This defines for most VDR sites and their target genes a chromosomal environment determining which gene is controlled by which receptor locus in its vicinity [94]. Transient sites, *i.e.*, VDR loci that are occupied only at certain time points after ligand stimulation, modulate the vitamin D responsiveness of the epigenome, *i.e.*, they support the actions of VDR molecules binding at persistent sites. Interestingly, the most critical attribute for the functional distinction of vitamin D-modulated TADs is the relative number of persistent and transient VDR sites [55]. For example, vitamin D target genes that are critical for immune function responding in an "on/off" modus and are primarily up-regulated by transient VDR loci (Fig. 1C) [94]. Persistent and transient VDR loci build up a specific local epigenomic environment that influences chromatin accessibility as well as histone modifications at TSS and enhancer regions. Thus, vitamin D-triggered changes of the epigenome and its consequences on the transcriptome are secondary effects of the activity of persistent and transient VDR sites acting on primary vitamin D target genes.

The chromatin model of vitamin D signaling (Fig. 1B) describes vitamin D-triggered changes of the association of VDR and pioneer factors with their genomic sites affecting chromatin accessibility [14,60]. This results in a significant modulation of the expression of one or more primary vitamin D target genes located within the same TAD [95]. This implies that epigenetic changes occurring during the process of tumorigenesis influence the response of vitamin D target genes. However, this mechanism also bears the potential that a sufficient vitamin D status of a cancer patient may lead to epigenetic changes within tumor cells that affect critical target genes, such as the up-regulation of the cell cycle inhibitor *CDKN1A* (cyclin dependent kinase inhibitor 1A) [96,97], resulting in growth arrest (Fig. 2B). Thus, vitamin D-triggered changes of the epigenome in the genomic region of the *CDKN1A* gene keep the gene actively transcribed and allow its encoded protein to act as inhibitor of the cell cycle *via* binding to cyclin-cyclin-dependent kinase (CDK) complexes.

Transcriptome-wide studies using microarrays and RNA sequencing (RNA-seq) in various cellular systems (including cell lines representing prostate, breast, ovarian, colorectal, squamous cell carcinoma and leukemia [66]) identified a large set of primary and secondary vitamin D target genes. The latter are genes that are not directly regulated by VDR but are up- or down-regulated *via* transcription factors, chromatin modifiers or non-coding RNAs (ncRNAs) that are encoded by primary vitamin D target genes. Some of these are regulators of the cell cycle [98–100]. Common cellular processes targeted by vitamin D are cell cycle progression, apoptosis, cellular adhesion, oxidative stress, immune function and steroid metabolism. The most comprehensive description of the vitamin D-modulated transcriptome, as determined by RNA-seq [101], was performed with leukemia cells (THP-1), which were stimulated for 2.5, 4 and 24 h with 10 nM $1,25(OH)_2D_3$ [79]. Statistics for differential expression initially indicated 1284 vitamin D target genes [79], while a re-analysis of this dataset reduced the number of genes to 587 [55]. A bit more than half (311) of these genes are primary targets of vitamin D. Gene ontology analysis indicated that immune functions, such neutrophil signaling and inflammatory responses, are the most significant process regulated by these primary vitamin D target genes [95].

Transcriptomic studies in human CRC have been performed in immortal carcinoma cell lines, in primary patient-derived NFs, in cancer-associated fibroblasts (CAFs) and in organoids generated by normal and cancer stem cells (Table 1). The first microarray analysis showed a regulation of hundreds of genes after 4 and 48 h of $1,25(OH)_2D_3$ treatment (10 nM) in SW480-ADH cells and a far lower number of genes in LS174 T cells [98]. Most target genes were up-regulated and their encoded proteins were preferentially involved in transcription, cell adhesion, DNA synthesis, apoptosis, redox status and intracellular

Table 1
$1,25(OH)_2D_3$ target genes in human colon and breast cancer cells. List of key representative genes regulated by $1,25(OH)_2D_3$ in human colorectal and breast cancer or stromal cells indicating the process and signaling pathway they are involved in.

Gene	Process/Pathway	Cell type	Cancer type	Refs.
MYC, JUN, JUNB, JUND, FOS…	Proliferation/transcription	Carcinoma cells	Colorectal	[56,98,100,145]
CCND1, CDKN1A, CDKN1B, G0S2…	Proliferation/cell cycle control	Carcinoma cells	Colorectal	[56,98]
EGFR, SPRY2, AREG	Proliferation/growth factor signaling	Carcinoma cells	Colorectal	[100,147,148,149]
CDH1, OCLN, CLD2, TJP1, CASR, FLNA…	Differentiation/cell adhesion	Carcinoma cells	Colorectal	[99,141]
KDM6B, other histone demethylases	Epigenetic regulation	Carcinoma cells	Colorectal	[83,152]
BAK, BAG, BIRC5, BAX, G0S2, IGFBPs	Apoptosis	Carcinoma cells	Colorectal	[98,138]
HIF1A, VEGF, DKK4…	Migration, angiogenesis, invasiveness	Carcinoma cells	Colorectal	[98,150,151]
CST5, DKK1	Proliferation, differentiation, migration…	Carcinoma cells	Colorectal	[142,153,155]
CYP3A4 SULT2A1, ABCC1…	Detoxification, metabolism	Carcinoma cells	Colorectal	[98,138]
Protease, protease inhibitors	Pleiotropic effects/protein half-life	Carcinoma cells	Colorectal	[98,155]
miR22	Proliferation, migration	Carcinoma cells	Colorectal	[156]
RELA, NFKB1A	Immunomodulation/transcription	Carcinoma cells, immune cells	Colorectal	[108]
IL1B, IL6, IL6, IL8, IL10, IL17, IL23	Immunomodulation/intercellular communication, cytokines	Carcinoma cells, immune cells	Colorectal	[108]
CCL2, CCL11, CCL13, CYTL1	Intercellular communication/chemokines	CAFs	Colorectal	[67]
NID2…	Extracellular matrix	CAFs	Colorectal	[67]
TIMP3, S100A4, CD82, SEMA3B	Migration, invasiveness, metastasis	CAFs	Colorectal	[67]
DKK4, TFF2, JSRP1, S100 P, TNS4, BCAS1, CA2…	Proliferation, invasiveness	Cancer stem cell-derived organoids	Colorectal	[57]
MYC, CCND1, CDK2, CDK4, CDKN1A…	Proliferation/cell cycle control	Carcinoma cells	Breast	[177]
CDH1, CDH2, CDH3	Differentiation/cell adhesion	Carcinoma cells	Breast	[175]
TIMP1, MMP9, TNC, ITGA6, ITGB4, VEGFA, ID1	Migration, angiogenesis, invasiveness	Carcinoma cells	Breast	[175,176,177]
BCL2 family	Apoptosis	Carcinoma cells	Breast	[172,173,174]
ESR1, CYP19A1	Proliferation/estrogen signaling	Carcinoma cells, stromal adipocytes	Breast	[178,179]
PTGS2, PTGER2	Proliferation/prostaglandin signaling	Carcinoma cells, stromal adipocytes	Breast	[178,179]

signaling. In another microarray study performed in Caco-2 adenocarcinoma cells 12 genes (including *CYP24A1* and others potentially involved in inhibition of cell proliferation) were found to be regulated upon 24 h treatment with 10 nM $1,25(OH)_2D_3$ [100]. A later microarray study in LS180 cells with a 24 h treatment (10 nM) identified further $1,25(OH)_2D_3$ target genes responsible for vitamin D catabolism, calcium and phosphate uptake, secondary bile acid metabolism and xenobiotic degradation and control of cell proliferation, such as *MYC* (MYC proto-oncogene, BHLH transcription factor) and *FOS* (Fos proto-oncogene, AP-1 transcription factor subunit)) [56]. As confirmed later by ChIP-seq analysis (3 h, 10 nM $1,25(OH)_2D_3$) also in other cell systems, the majority of potential regulatory VDR binding sites are located far from the TSSs or in introns of their target genes.

When considering the putative relevance of these transcriptomic studies, it is convenient to remind that cell lines used have complex and heterogeneous mutational landscapes following long-term culture. Moreover, in some cases these cell lines, such as Caco-2 cells, which are of colon origin but shows the phenotype of small intestinal epithelium, do not properly model the correct type of cancer.

A strong regulatory effect of $1,25(OH)_2D_3$ has been found in human colon stromal fibroblasts in a microarray study (48 h, 10 nM $1,25(OH)_2D_3$) highlighting 958 genes (47 % up-regulated, 53 % down-regulated) in NFs and even 1489 genes (35 % up-regulated, 65 % down-regulated) in CAFs [67]. These gene lists partially overlap and most of the genes are involved in cell adhesion and migration, extracellular matrix organization, blood vessel development, inflammatory response and cell communication. In an RNA-seq study in the immortal CCD-18Co human colon fibroblast cell line, $1,25(OH)_2D_3$ (24 h, 10 nM) and WNT3A (WNT family member 3A) showed additive, partially overlapping effects on the transcriptome. Curiously, both agents inhibit the proliferation and migration capacities of CCD-18Co cells, while $1,25(OH)_2D_3$ reduces but WNT3A increases the ability to contract collagen gels. The latter is a surrogate marker of the capacity to alter the extracellular matrix, which is a hallmark of fibroblast activation. These effects were reproduced in patient-derived NFs and CAFs as well as in fibroblastic cell lines of several origins suggesting that $1,25(OH)_2D_3$ and WNT3A regulate the gene expression and phenotype of human colon fibroblasts [102].

In patient-derived colon tumor organoids, a 96 h treatment with 10 nM $1,25(OH)_2D_3$ changes the RNA level of 1182 genes (643 up-regulated, 539 down-regulated), while in matched (same patient) normal organoids generated from healthy colon tissue the number of target genes was high as 2107 (943 up-regulated, 1164 down-regulated) [57]. Only 19.3 % of the genes in these lists overlap, which indicates that $1,25(OH)_2D_3$ regulates distinctly genes, *e.g.*, related to cell stemness and differentiation, in the two types of organoids. In these and other transcriptomic studies with $1,25(OH)_2D_3$ over longer time periods, approximately half of the target genes are down-regulated, *i.e.*, the level of their RNA transcripts are diminished by $1,25(OH)_2D_3$. Gene repression by ligand-modulated nuclear receptors, such as VDR, is primarily an indirect and/or post-transcriptional effect mediated by the products of primary target genes, such as transcription factors, chromatin modifiers and ncRNAs, through protein-protein interactions, signaling interference or microRNAs (miRs). In contrast, in a process referred to as transrepression ligand-induced VDR directly represses primary vitamin D target genes, such as *PTH* (parathyroid hormone) in the parathyroid gland [103] and *CYP27B1* in the kidneys [104].

Taken together, $1,25(OH)_2D_3$ significantly changes the transcriptome of healthy and malignant cell types at 1000–2000 genomic loci. Longer VDR ligand treatments lead to a higher percentage of secondary vitamin D target genes. However, a weakness of these studies is the heterogeneity of the cell types, their treatment protocols, such as number of replicates, duration and doses of $1,25(OH)_2D_3$, and the statistical analyses making it difficult to translate their results to the identification of target genes.

6. Impact of the immune system on the anti-cancer role of vitamin D

There is large consensus that the modulation of the immune system is the most important extra-skeletal function of vitamin D [105,106]. Vitamin D stimulates the innate immune system in fighting more efficiently against bacterial infections, such as tuberculosis [105], while it prevents overreactions of the adaptive immune system that may cause

autoimmune diseases, such as multiple sclerosis [107,108]. On the basis of transcriptome-wide datasets from THP-1 cells (monocytes) and human peripheral blood mononuclear cells (PBMCs, a mixture of lymphocytes and monocytes) treated *in vitro* with $1,25(OH)_2D_3$ as well as from PBMCs of individuals supplemented with a vitamin D_3 bolus three groups of immune-related vitamin D target genes had been identified that represent the functions acute response to infection, infection in general and autoimmunity [109]. Interestingly, the vitamin D target genes can be differentiated in their epigenomic profile, such as the types of VDR-driven enhancers, as well as *via* the dynamics of their mRNA production.

In general, vitamin D acts as an inducer of innate immunity, such as *via* the prominent up-regulation of the secreted anti-microbial peptide cathelicidin or plasma membrane-anchored glycoprotein CD14 [110]. CD14 functions as a co-receptor for the pattern recognition receptors Toll-like receptors (TLRs) [111] and delivers the pathogen-associated molecular pattern lipopolysaccharide to TLR4. Thus, the early response of monocytes and macrophages to vitamin D stimulation is a pro-inflammatory action [112]. In a later step vitamin D often shifts the polarization of macrophages from the pro-inflammatory, anti-tumor stage M1 to the immunosuppressive, pro-tumor M2 stage [113]. However, please note that the stages M1 and M2 are considered the extremes of a wide spectrum of plasticity of macrophages that is sensitive to epigenetic programing by vitamin D and its receptor VDR [60,114]. Thus, the anti-inflammatory and immunosuppressive functions of vitamin D are paired in certain cases with a pro-tumor effect.

Vitamin D deficiency is associated with Crohn's disease and ulcerative colitis, which are the two predominant pathophysiological manifestations of inflammatory bowel disease [115,116]. The rates of inflammatory bowel disease are probably increasing due to modern lifestyles that affect the function of the gut microbiome *via* high levels of saturated fat and sugar in diet as well as the use of antibiotics [117]. Vitamin D is important for regulating the immunity of gut mucosa *via* the modulation of innate immune barrier function, gut epithelial integrity and the development and function of T cells [118,119]. Thus, vitamin D can prevent the onset of inflammatory bowel disease through the stabilization of microbiota homeostasis as well as ameliorates disease progression *via* anti-inflammatory immune responses. Interestingly, in persons with increase susceptibility for inflammatory bowel disease vitamin D deficiency is a contributing factor to the disease [120].

Knockout mice for *CYP27B1* [121] as well as for *VDR* [122] show increased severity of experimentally-induced colitis that mimics inflammatory bowel disease, *i.e.*, the inability to synthesize or recognize $1,25(OH)_2D_3$ increases the severity of inflammatory bowel disease. Moreover, a treatment with $1,25(OH)_2D_3$ improves the conditions of experimental colitis in murine models [115,123]. Accordingly, a low vitamin D status is a significant risk factor for the onset of CRC in individuals with inflammatory bowel disease [124].

Vitamin D mostly functions as a repressor of adaptive immunity, as it down-regulates the number of T_H (T helper) 1 cells and increase that of T_H2 cells and T_{reg} (T regulatory) cells. However, the majority of these observations were made in experimental settings in which the cells were overstimulated. Thus, vitamin D may have a late role accelerating the resolution of inflammation following an early role in its initial activation [108,125–127]. Interestingly, a human intervention trial indicated that in the context of Western diet vitamin D_3 supplementation increases the expression of inflammatory genes in colon cells, while additional supplementation with calcium reverses this effect [128]. This may explain how a sufficient vitamin D status combined with high dietary calcium intake is linked to lower CRC risk.

Vitamin D actions on the immune system involve the growth, differentiation, activation/deactivation and eventually apoptosis of different types of immune cells, such as monocytes, dendritic cells and different types of T cells [129]. Importantly, immune and cancer cells use the same signal transduction pathways and genes for pushing their growth [130]. Therefore, it is possible that the anti-proliferative and differentiation- and apoptosis-inducing potential of vitamin D on cancer cells derives from pathways that were initially evolved for the growth control of immune cells, such as the up-regulation of the *CDKN1A* gene [131]. Moreover, these cell types are also found in the microenvironment of tumors, *i.e.*, some aspects of the anti-cancer effects of vitamin D could be explained by a modulation of the immune component of the microenvironment, which may be detrimental for cancer cells [132,133].

Tumor cells present at their plasma membrane antigens that can be specifically recognized by therapeutic monoclonal antibodies, such as Rituximab (anti-CD20) in B-cell lymphomas and Trastuzumab (anti-HER2, human epidermal growth factor receptor 2) in breast cancer. Vitamin D deficient patients are poor responders to this therapy [134,135]. This suggests another mechanism of $1,25(OH)_2D_3$ with a potential anti-tumoral relevance in the clinic, which is the enhancement of the antibody-dependent cellular cytotoxicity of macrophages and natural killer cells. Nevertheless, the main impact of the immune system in explaining the anti-cancer effect of vitamin D may not be in controlling and reducing settled tumors but in preventing their establishment. In an healthy individual every day thousands of cells undergo alterations that change from a normal to a malignant phenotype, but most of them are detected in this early stage by cytolytic T cells and destroyed [136]. Thus, an activation of cytolytic T cells may be an effective way how vitamin D prevents cancer onset [137].

In summary, there are many mechanisms through which the prime action of vitamin D, the modulation of innate and adaptive immunity, affects the growth, differentiation and apoptosis of cancer cells and explains the anti-cancer action of the nuclear hormone.

7. Vitamin D and CRC

A large number of mechanistic studies *in vitro* as well as in experimental animals support the beneficial action of VDR agonists, which had been suggested by epidemiology data that linked vitamin D deficiency to high incidence and/or mortality by CRC [138]. The regulation of more than thousand vitamin D target genes in colon carcinoma cells, normal stem cells, cancer stem cells, stromal NFs and CAFs as well as immune cells of the tumor microenvironment in combination with the tumor-inhibitory effects of vitamin D in xenografted mice strongly indicate a protection against this form of neoplasia (Fig. 3).

CRC is the best characterized solid neoplasia in terms of genetic alterations. Massive sequencing efforts have shown that over 94 % of primary and up to 96 % of metastatic colorectal tumors contain mutations in genes that aberrantly activate the WNT/β-catenin signaling pathway [139,140]. Thus, mutually exclusive mutations of the genes *APC* (APC regulator of WNT signaling pathway), *CTNNB1* (catenin beta 1) and *AXIN2* (axin 2) and less frequently of *RSPO2/3* (R-spondin 2 and 3), *RNF43* (ring finger protein 43) and *TCF7L2* (transcription factor 7 like 2) in colon epithelial (probably stem) cells are considered the initiation of the tumorigenic process.

$1,25(OH)_2D_3$ has two major effects on colon carcinoma cells: it inhibits the proliferation and promotes the differentiation through several mechanisms including i) the induction of VDR binding to β-catenin within the cell nucleus that prevent the formation of transcriptionally active TCF7L2/β-catenin complexes, ii) the up-regulation of the protein CDH1 (cadherin 1, also called E-cadherin) at the plasma membrane where it attracts the newly synthesized β-catenin protein and iii) the induction of the gene *DKK1* (Dickkopf WNT signaling pathway inhibitor 1) [141–143].

An important anti-proliferative mechanism of $1,25(OH)_2D_3$ is the inhibition of the *MYC* gene, which is a major cell cycle regulator being induced by the WNT/β-catenin pathway and over-expressed in CRC as well as in many other cancer types. While nearly all CRCs have changes in MYC target genes, vitamin D represses the *MYC* gene directly by VDR binding to its promoter [144] and indirectly *via* inhibition of the WNT/

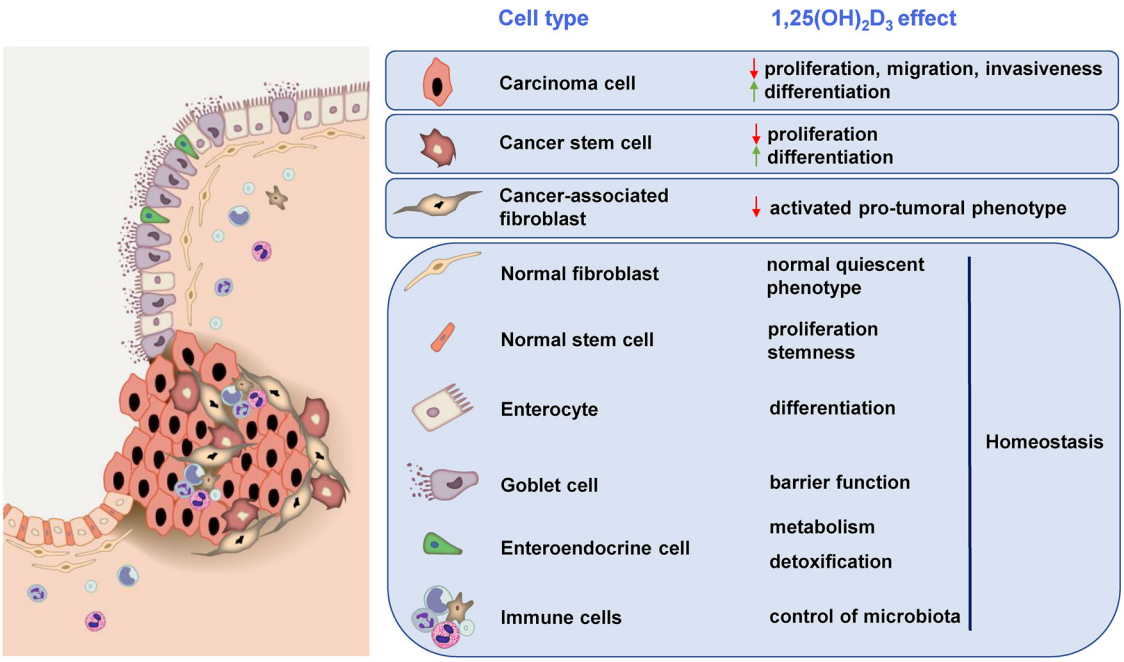

Fig. 3. Vitamin D effects in CRC. The scheme shows the topographic distribution of the cell types in the colon crypt. Effects of $1,25(OH)_2D_3$ on the different cell types (tumor and stromal) in a colon neoplasia and in adjacent normal tissue. Homeostatic effects on normal cells and protective effects on tumor cells are indicated.

β-catenin pathway [141], the induction of its antagonistic partner MAD/MXD1 of MYC [145] and the enhancement of its FBW7 E3 ligase-mediated ubiquitination and subsequent proteasomal degradation of the MYC protein [146]. $1,25(OH)_2D_3$ regulates also other genes encoding cell cycle proteins or members of mitogenic pathways (Table 1). Another mode by which $1,25(OH)_2D_3$ reduces cell proliferation is the interference of signaling by mitogens. Vitamin D reduces the expression and activity of the *EGFR* gene and also represses the *SPRY2* (sprouty RTK signaling antagonist 2) gene, which encodes an activator of intracellular EGF signaling [147–149]. Likewise, $1,25(OH)_2D_3$ inhibits signaling by insulin growth factor (IGF) 2 protein.

These findings show that $1,25(OH)_2D_3$ is a multi-level inhibitor of colon carcinoma cell proliferation *via* controling a variety of genes and several mitogenic signaling pathways. Conceivably, this anti-tumoral mechanism is a secondary effect of the evolutionary selection of $1,25(OH)_2D_3$ as a homeostatic regulator of the organism through the control of a large proportion of the human genome. Supporting this idea, in colon carcinoma cells $1,25(OH)_2D_3$ also regulates genes involved in apoptosis, angiogenesis and migration/invasiveness as well as in cell adhesion and differentiation (Table 1) [98,150,151].

Remarkably, epigenetic regulators, such as *KDM6B* and other histone demethylases [83,152] as well as *CST5* (cystatin D) are also under the control of $1,25(OH)_2D_3$ [153,154]. *CST5* is a member of the cathepsin gene family encoding a multifunctional protein with protease activity in lysosomes and gene regulatory action within the cell nucleus. Moreover, several genes encoding enzymes and inhibitors of the ubiquitin-proteasome system are modulated by vitamin D in colon carcinoma cells suggesting that $1,25(OH)_2D_3$ has a role in the post-translational control of gene expression [155]. Additional pathways affected by liganded VDR are detoxification and immunomodulation-related signaling, the latter probably both in carcinoma cells and in immune cells of the tumor microenvironment (Table 1) [102,108].

An indirect but potent mechanism of controling the phenotype of colon carcinoma cells by $1,25(OH)_2D_3$ is the regulation of miR-22, which contributes to the anti-proliferative and migratory actions of vitamin D and has also anti-tumor activity in several cancers [156].

The induction of an epithelial differentiated phenotype is a second major effect of $1,25(OH)_2D_3$ in colon carcinoma cells. A key mechanism is the up-regulation of the *CDH1* gene [121], the encoded protein of which is the main component of adherens junctions being the most important structure responsible for intercellular adhesion in epithelia. The promotion of strong cell-to-cell adhesion, which reduces their capacity to proliferate, is further supported by the induction of other genes, *OCLN* and *TJP1*, coding for components of additional adhesion structures, such as tight junctions and desmosomes [121] (Table 1).

Remarkably, a detailed study of the induction of the *CDH1* gene revealed a rapid, non-genomic signaling pathway triggered *via* extranuclear VDR [157]. $1,25(OH)_2D_3$ increases cytosolic Ca^{2+} concentration in a transcription-independent way by favoring its entry from the external medium, which causes the cascade activation of RHOA (Ras homolog family member A), ROCK1 (Rho associated coiled-coil containing protein kinase 1) and the kinases MAPK14 (mitogen-activated protein kinase 14) and RPS6KA5 (ribosomal protein S6 kinase A5). Importantly, the induction of this pathway is necessary for the gene regulatory action of $1,25(OH)_2D_3$ within the cell nucleus in a variety of cell types. This indicates a dual role of VDR and the convergence of non-genomic and genomic $1,25(OH)_2D_3$ signaling pathways [157]. Thus, $1,25(OH)_2D_3$ has a key role in the biology of colon epithelial cells counteracting the genetic and epigenetic alterations that promote the apparition of colon carcinomas.

A large proportion of advanced CRC cases do not express the *VDR* gene, which is due to the up-regulation of the transcription factors SNAI1 (snail family transcriptional repressor 1) and SNAI2 that bind and block the *VDR* promoter region [158,159]. This suggests that VDR agonists have a protective effect in the prevention and early stages of CRC. However, a recent study [67] analyzing more than 600 CRC biopsies revealed that high VDR expression in CAFs associates with better clinical patient outcome. Moreover, $1,25(OH)_2D_3$ imposes in primary CAF cultures a gene signature (48 genes) that is associated with longer patient survival [67]. Accordingly, $1,25(OH)_2D_3$ regulates in these cells signaling pathways that are linked to a phenotype of activated fibroblasts by modulating of extracellular matrix and cytokine/chemokine-mediated cell migration and communication. As a result, $1,25(OH)_2D_3$ attenuates two pro-tumoral effects of CAFs, which are their capacity to contract/alter the collagen gel and to promote the migration of carcinoma cells [67]. $1,25(OH)_2D_3$ reprograms stromal fibroblasts towards a less pro-tumoral phenotype as already shown for liver and pancreas cancer [132,160]. This indicates that the beneficial

actions of VDR agonists also affect stromal cells of the tumor microenvironment. Thus, CRC patients with VDR-negative carcinoma cells may still benefit from vitamin D treatment.

Based on the concept of Tomasetti and Vogelstein [161] 2/3 of all cancer mutations are based on replication errors, primarily of normal adult stem cells. A large part of these mutations are due to extrinsic factors, *i.e.*, they are based on lifestyle decisions, such as smoking and diet choice. However, until recently the effect of vitamin D on non-hematopoietic stem cells had not been studied. Therefore, a recent study [57] using organoids generated with healthy colon tissue from CRC patients became very important, as it indicated that $1,25(OH)_2D_3$ inhibits cell proliferation and maintains the stem cell phenotype by increasing the expression of several stemness-related genes (*LGR5* (leucine rich repeat containing G protein-coupled receptor 5), *SMOC2*, *LRIG1*, *MEX3A*, *MSI1*, *PTK7*). In contrast, in matched tumor organoids of the same patients $1,25(OH)_2D_3$ promotes cell differentiation with only minor changes in stemness genes. Moreover, vitamin D also causes a variable inhibitory effect on cell proliferation and the repression of genes linked to the tumorigenesis process. In contrast to carcinoma cells with an overactivated WNT/β-catenin pathway, $1,25(OH)_2D_3$ does not affect WNT target genes in stem cell-derived organoids. In the latter, this signaling pathway plays a major role in the maintenance of stemness with EGF acting as the main mitogen. Thus, $1,25(OH)_2D_3$ contributes to the preservation of the undifferentiated phenotype of crypt bottom adult stem cells and their basal level of proliferation, but, importantly, it attenuates their transformation into cancer stem cells. For example, feeding mice a purified rodent Western-style, low vitamin D diet perturbs intestinal cell maturation and WNT signaling and causes sporadic colon and small intestinal tumors. The diet acts as extrinsic factor and associates with extensive transcriptional reprograming, such as decreasing the number of mouse $Lgr5^{high}$ intestinal stem cells [162–164], and supports the role of vitamin D in controlling intestinal stem cells. Thus, the anti-cancer effect of vitamin D, in particular on CRC, may be primarily caused by its effects on adult stem cells.

Taken together, vitamin D modulates an ample array of signaling pathways in the diverse cell types involved in CRC (Fig. 3 and Table 1). Both the homeostatic nature of those promoted in normal adult stem cells and NFs and the anti-tumor nature of those induced in carcinoma cells, CAFs and cancer stem cells strongly indicate a strong, multi-level protective action of vitamin D against CRC.

8. Vitamin D and breast cancer

Data on the association of vitamin D and breast cancer incidence and/or mortality are inconclusive and less clear than for CRC [165]. However, two recent meta-analyses suggest a protective relationship between $25(OH)D_3$ serum levels (not vitamin D intake, emphasizing the importance of individual genetics and metabolism) and breast cancer risk [166,167]. Some studies [166], but not others [167], have found a preferential effect in pre-menopausal women. The controversial results reported may be, at least in part, due to the heterogeneity of breast cancer, which comprises several pathological and genetic subtypes. Interestingly, the triple-negative breast cancer (TNBC) subtype, which is characterized by the lack or low tumor expression of ER, PR and HER2 protein (ER^-, PR^-, $HER2^-$) and a poor prognosis, may preferentially benefit from the protective effect of vitamin D [167,168]. Supporting this, TNBCs showing positive VDR staining correlate inversely with bad pathological features and directly with increased overall survival [169].

As for CRC, studies in cultured cells and animal models of breast cancer support a protective effect of VDR agonists, which appears to result from direct effects on tumor cells rather than on systemic actions or effects on stromal cells of the microenvironment [170]. Thus, treating breast cancer cell lines with $1,25(OH)_2D_3$ induces anti-proliferative [171] as well as pro-apoptotic effects [172,173]. The former is linked to the suppression of growth stimulatory signals and genes (*MYC* and genes coding for cyclins and CDKs) and the potentiation of growth inhibitory signals and genes, whilst the latter is explained by BCL2 (BCL2 apoptosis regulator) family proteins. Moreover, $1,25(OH)_2D_3$ favors the differentiated, non-invasive epithelial phenotype of breast tumor cells by both inducing genes, such as *CDH1*, while repressing those encoding for N- and P-cadherins (*CDH2* and *CDH3*) and others [174–176] (Table 1). Thus, as in CRC, *MYC* and *CDH1* seem to be crucial target genes of $1,25(OH)_2D_3$ in breast carcinoma cells.

Other studies, however, proposed tumor non-autonomous effects of vitamin D on breast cancer. For example, $1,25(OH)_2D_3$ reduces the adverse effects of obesity on breast cancer by acting on pathways both within breast cancer cells and surrounding adipocytes by suppressing estrogen synthesis and signaling *via* the control of prostaglandin E_2 synthesis by PTGS2 (prostaglandin-endoperoxide synthase 2) and degradation, and the inhibition of ER and PTGER2 (prostaglandin E receptor 2) and CYP19A1 (aromatase) [177,178]. Giving the important role of estradiol promoting the proliferation of breast cancer cells at least at early stages of tumor development, these effects on estrogen signaling may protect against breast cancer at the initial steps. In addition, $1,25(OH)_2D_3$ enhances AMPK (adenosine monophosphate-activated protein kinase) signaling and restores the adipokine profile by decreasing leptin signaling and stimulating adiponectin signaling that are dys-regulated in response to diet-induced obesity in both tumor cells and surrounding adipose tissue.

Finally, $1,25(OH)_2D_3$ inhibits spheroid formation by mouse breast tumor-initiating cells by down-regulating ER [179]. Likewise, $1,25(OH)_2D_3$ inhibits mammosphere formation in cultures of MCF10DCIS and SUM159 breast cancer cell lines by down-regulating the expression of stem cell markers and several NOTCH pathway genes [180].

In summary, the effects of vitamin D on breast cancer are partially similar to but overall less convincing than those on CRC. Moreover, the effect, if any, of $1,25(OH)_2D_3$ on authentic human breast cancer stem cells remains to be elucidated.

9. Vitamin D and prostate cancer

The association between vitamin D status and prostate cancer is much less compelling than those for colorectal or breast cancer suggesting that not all tissues or cancer cell types respond identically. While some studies found a direct relation between circulating $25(OH)D_3$ and prostate cancer risk or mortality, others did not, and even a few reported that high levels of $25(OH)D_3$ are associated with increased risk, altogether leading to inconclusive meta-analyses [181,182]. A limited number of interventional clinical trials using vitamin D compounds as single agents or in combination have been seriously criticized for bad design and flawed conclusions [183]. Still, data from pre-clinical studies on the effect of vitamin D in prostate cells and animal models do not differ much from those in other cancer types. In addition to the usual anti-proliferative and apoptotic-sensitization effects, $1,25(OH)_2D_3$ has in prostate cancer regulatory actions on *PTGS2* and other genes of the prostaglandin pathway as well as on IL6 suggesting an anti-inflammatory effect [184]. Moreover, $1,25(OH)_2D_3$ activates in prostate cancer cells metabolic enzymes, such as CYP3A4, CYP3A5, UGT2B15/17 and SULT2B1, that may reduce the availability of androgens acting as mitogens in these cells.

A microarray study in the human non-transformed RWPE1 prostate epithelial cell line showed that $1,25(OH)_2D_3$ regulates a high number of genes suggestive of the suppression of cancer- and inflammation-related signaling pathways (WNT, NOTCH, NF-κB, IGF1, IL1, IL6, IL17) and the induction of protection from oxidative stress [185]. Concordantly, in the $TgAPT_{121}$ mouse model of prostate cancer dietary vitamin D reduces the apparition of adenocarcinomas whereas the loss of the *Vdr* gene accelerates the early stages of prostate carcinogenesis [53]. In this system, microarray and ChIP-seq analysis identified thousands of target genes, some of which are involved in cell cycle and proliferation (*FOS*,

EGR1, *MAPK8*, *HGF*, *MYC*), apoptosis (*FOXO1*, *SRF*, *CALM2*), cell adhesion (*EPHB1*, *EPHA2*, *MLCK*), immune response (*CCL16*, *IL6*, *IFNG*) and gene transcription/expression (*HIF1A*, *SMAD3*, *CREB1*, *CREM*, *CBF1, ESR1*). Interestingly, in another model of prostate cancer, TRAMP mice, early treatment with vitamin D slowed androgen-stimulated tumor progression by reducing tumor cell proliferation and inducing E-cadherin but in long-term increased distant organ metastasis [186].

Taken together, prostate cancer is complex with an intricate hormonal regulation and multiple cell types involved. Still much work is needed to elucidate the mechanisms and potential utility of vitamin D in this neoplasia.

10. Vitamin D and hematological malignancies

As it happens in solid tumors, patients with hematological malignancies, such as leukemias, lymphomas and multiple myeloma, usually have a low vitamin D status being associated with a poorer prognosis [187–190]. As in other cancer cell types, VDR agonists inhibit proliferation (AML, B cell acute lymphoblastic leukemia, follicular non-Hodgkin's lymphoma), sensitize to apoptosis promoted by signals including other anti-tumor therapies (AML, cutaneous T lymphoma, multiple myeloma, diffuse large B cell lymphoma, B cell chronic lymphocytic leukemia), induce differentiation (AML, follicular non-Hodgkin's lymphoma) and usually decrease the production of pro-inflammatory cytokines [191]. $1,25(OH)_2D_3$ decreases the activation of the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) signaling pathway, as revealed by diminution of the level of phospho-STAT3 and phospho-STAT1. This pathway is commonly overactivated in leukemia and lymphoma cells due to mutation in JAKs or STATs or by abnormally high cytokine signaling. As a consequence, $1,25(OH)_2D_3$ decreases the production of the cytokines IFNG (interferon gamma), TNF (tumor necrosis factor) and several interleukins [192].

AML is a heterogeneous group of leukemias that result from clonal transformation of hematopoietic precursors through the acquisition of chromosomal rearrangements and multiple gene mutations. As AML cells are proliferatively immature, differentiation therapy is potentially effective for initiation of terminal differentiation and represents a supplementary approach for the treatment of neoplastic disease in combination with existing treatment modalities [193]. Importantly, $1,25(OH)_2D_3$ initiates differentiation in AML cell lines, such as HL60, which are predominantly neutrophilic promyelocytes. Treatment of HL60 cells with $1,25(OH)_2D_3$ results in a reduced proliferation and enhanced differentiation along the monocyte-macrophage pathway possibly by mechanisms involving the up-regulation of *CEBPD* [194,195]. Moreover, together with the pioneer factors CEBPA and PU.1 VDR is the key regulator of the differentiation of monocytes and granulocytes [88] probably *via* the induction of the *CDKN1A* gene [196]. Interestingly, in a zebrafish model it had been shown that vitamin D regulates the number of embryonal hematopoietic stem cells [44]. This observation was confirmed *ex vivo* with human hematopoietic stem cells possibly *via* the induction of the gene *CXCL8* (C-X-C motif chemokine ligand 8) that is known as a primary vitamin D target [197]. This suggests that vitamin D first pushes the growth of hematopoietic stem cells and then induces their differentiation into myeloid cells. Accordingly, in the context of hematopoietic stem cell transplants vitamin D deficiency is known to be associated with increased complications, such as graft-*versus*-host disease, delayed time to neutrophil engraftment and overall survival [198,199].

A recent mice study showed that *Vdr* deficiency in hematopoietic precursors causes an increase in the expression of the genes *MECOM* (MDS1 and EVI1 complex locus), *HOXA9* (homeobox A9) and *MEIS1* (meis homeobox 1) suggesting the induction of an immature phenotype and, concordantly, of the number leukemia stem cells and normal hematopoietic stem cells [200].

In summary, VDR is a new genetic modifier contributing to the expression of different subtypes of acute myeloid leukemia, *i.e.*, vitamin D plays an important role in normal but also in pathological hematopoiesis.

11. Conclusion

Epigenomic and transcriptomic analyses as well as numerous experimental studies in a variety of cancer systems provided large sets of strong data that jointly indicate a protective action of vitamin D signaling against several types of cancer. Most convincing molecular data exist for CRC and AML. This results from the regulation of a high number of genes that control the proliferation, survival, differentiation, migration and communication of cancer cells and stromal cells, such as fibroblasts, adipocytes, immune and endothelial cells. Conceivable, the lack of concordant positive clinical data in large randomized controlled trials may be due to lack of sub-analysis of disease cohorts (*e.g.*, the selection and analysis of patients) as well as short follow-up. Moreover, the careful characterization of disease subgroups should lead to more consistent data as shown for TNBC. Vitamin D is a major regulator of signaling in human cells, which displays a long list of protective homeostatic effects in cultured cells and animal models of cancer. Thus, it contributes to maintain and defend the normal physiology of the organism against the apparition and development of neoplasias. The identification of the optimal clinical use of the vitamin D system is a task demanding continuous efforts.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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